Application type: Patent of Invention
Application number: 01-2086
Application Date: Monday, August 27, 2001
Priority Number: none

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Title: Highly immunogenic protein against the intracellular pathogenic *Piscirickettsia salmonis*, which affects salmon cultures. The amino acid and nucleic acid sequences of said protein and its use in the development of methods useful for the prevention and diagnosis of diseases caused by said pathogenic

Abstract: A highly immunogenic protein against the intracellular pathogenic agent *Piscirickettsia salmonis*, is purified. Additionally, said protein is characterized by its amino acid and nucleotide sequences, which in turn are useful for obtaining the protein trough genetic engineering and for obtaining diagnosis kits and molecular markers for the diagnosis of this pathogenic . For preventing the infection of the salmonids with the pathogenic agent *Piscirickettsia salmonis*, a vaccine is developed.

DESCRIPTION

In general, the present invention belongs to the molecular biology area and more particularly to the area of biotechnology, since in its main aspect it describes the obtainment of useful tools for the prevention and diagnosis of the disease caused by *Piscirickettsia salmonis*; wherein said tools are obtained from a nucleic acid sequence and an amino acids sequence.

BACKGROUND OF THE INVENTION

Breeding of live species under captivity to produce meat aimed at direct human consumption, as is the case of salmon culture, represents an activity which is submitted to increasing requirements by consumers, requiring high quality standards, a decisive aspect for competing and remaining in the national and international markets.

The biological subjects, which are relevant for the production of salmonidae species, have been focused on the fish reproduction, diseases and feeding. Some aspects obtain almost immediate solutions devised by the producing companies, while others require both basic and advanced scientific research.

Nowadays, Chile is the second world producer in the salmonidae market with a total production and exportation of about 240,000 tons of salmon. An essential aspect of the culture, to control in the final product quality and the companies profitability, correspond to diseases, which often attack the fish population in a

heap up condition. In view of the importance of this market in our country, the solutions for combating bacterial and viral agents, which have a strong negative impact on aquaculture, should be addressed to the discovery of preventive treatments, such as vaccines.

Early in 1989, was described the Salmon Rickettsial Syndrome ("SRS") or piscirickettsiosis and since then it has caused significant mortality in salmonid aquaculture. This bacteria, causes the most important disease affecting the domestic salmon-producing system, representing the pathology that induces the greatest economic losses in Chile. Data supplied by various companies and national entities, indicate that the losses resulting from this microorganism's action, would reach figures close to US$ 80,000,000, which doubtless reflects the importance as a pathogenic agent of this bacterium.

The integrated control of diseases in salmons covers a number of measures that must be applied jointly. This involves monitoring, fast and accurate diagnose techniques, alternate use or short-term use of the culture locations, a suitable infrastructure offering genetic improvement, optimum hygiene and sanitation of the facilities; nutritional quality of the food, adequate feeding systems, careful handling of the fish ova from safe origin and a continuous technical capability. Furthermore, by way of important preventive measures against infection, the market offers techniques and products, such as immunostimulants, hygiene prophylaxis, antibiotics and integrated controls of

salmon diseases, specially emphasizing the support to the fish vaccine-based defensive system.

Glucanes, vitamin C and biogenic stimulators represent the immunostimulant substances. Immunostimulants have been tested just recently; therefore, there is no ready market for them. Both, the hygiene prophylaxis and the integrated disease control systems are a substitute based on actions aimed at prevention, however, they do not guarantee stopping the fish contagion by bacteria or virus.

Antibiotics, i.e., oxolynic acid, erythromycin, flumequine and oxitetracycline correspond to the only set of specific products for curative and non-preventive control of diseases that have been always applied to the salmonidae culture.

The sanitary prophylaxis corresponding to those hygiene-sanitary nature measures throughout the whole process chain of salmon production is based on sanitary measures, using appropriate methods and disinfectants which have a proven action against pathogens, additionally a healthy ova supply is carried out as a control.

A National Health Plan, elaborated by the Salmon Technological Institute has been implemented in Chile. This Plan is oriented at standardizing and supervising sanitary handling practices, and at obtaining and organizing the information provided by the industry in those areas that are sensitive to the company's productivity. Its action lines are the surveillance, prevention, diagnosis and diseases control.

All the above-mentioned substitute products are not sufficient to ensure the eradication of those infectious focuses of *Piscirickettsia salmonis* and of other bacteria and virus. The preventive management of diseases through the use of vaccines constitutes the single viable option; a situation that has been demonstrated by the strategy involving the eradication of antibiotics use and the eradication of other products applied at a domestic level in Norway, thereby provoking the full substitution of the same by vaccines.

Vaccines, in general, have the advantage of corresponding to a disease preventive management, whereby they would replace the antibiotics use, and not *vice versa*, additionally, vaccines have the advantage of showing an effective efficiency, an important and demonstrated costs reduction, long-lasting effects and a real preventive capacity, thus, additionally, their use overcomes all the disadvantages inherent in the antibiotics use.

Nowadays, vaccines for *Piscirickettsia salmonis* are commercially available. A commercially available vaccine Ricketvac®, which is prepared with duly characterized and titrated *Piscirickettsia salmonis* isolates, obtained *in situ*, propagated in cell cultures and formaldehyde-inactivated.

The fact of being dependant from propagating full bacteria cell cultures, implies having stringent sterility conditions and quality controls, thus at the industrial level such factors make difficult its production management. According to the prior art, which exhibits difficulties in the massive production of a vaccine from bacteria isolates, one objective of the present invention is to purify a highly

immunogenic protein against the intracellular pathogenic *Piscirickettsia salmonis*.

Another objective of the present invention is to characterize said protein through its aminoacid sequence and nucleotide sequences; in turn, said sequences will be useful toward obtaining the protein by means of genetic engineering.

A very important objective of the present invention is the development of a vaccine for preventing the infection of salmonidae species, by the *Piscirickettsia salmonis* pathogen agent.

Therefore, obtaining a vaccine both from the protein as well as from its DNA are considered objectives.

A further objective is to provide an efficient tool for the detection of *Piscirickettsia salmonis*, which is an important aspect of any integrated control program of the disease caused by this pathogenic .

The vaccine, according to the present invention, is distinguished by its high effectiveness in preventing the salmonidae contagion with *Piscirickettsia salmonis*, exhibiting much higher levels regarding those currently obtained using other products, and without exhibiting undesirable side effects, such as adherence.

For the *Piscirickettsia salmonis* vaccine, it can be concluded that there is a wide market, which broadly can compete with those currently existing vaccines, according to what will be demonstrated herein below. Vaccines have no real

substitutes. On the contrary, this product will substitute the use of antibiotics, which represent chemical compounds whose soonest strategic removal from salmon culture activities has already been proposed.

DETAILED DESCRIPTION OF THE INVENTION

The *Piscirickettsia salmonis* was purified from the supernatant of an infected cell culture, isolating its components by means of density gradients.

In order to generate antibodies against the surface proteins, the purified bacteria were injected in rabbits. Monodimensional profile protein extracts were disssolved in polyacrylamide gels from fish suffering from piscirickettsiosis, subsequently, the gels were transferred to nitrocellulose membranes. In parallel, these extracts were subjected to isoelectric focusing and then they were run in a second dimension in 10% polyacrylamide-SDS laminate gels, and they were also transferred to nitrocellulose membranes. The Western blot assay was completed for both membranes, with the above antibodies with similar molecular weights and isoelectric points; protein (A) of 59 kDa mass and 4.9 electrofocusing and (B) of 58.5 kDa mass and 4.8 electrofocusing.

Additionally, the extracts from diseased fish were assayed, being identified by dying the total proteins, 334 proteins were thus observed. Those proteins that react to the antibody were detected by overlapping in relation to all the proteins contained. Separately, the interesting proteins were eluted from pieces of gels and they were sequenced.

A similarity search was performed with the peptide sequences thus obtained, using the software Blastp from NCBI. The software found a high similarity percentage in both peptides, from 80% to 100%, using the GroEL, HSP60 chaperonin proteins, from several Proteobacteria species from the Gamma subdivision.

As a response to stress conditions, the bacteria produce a wide range of specific proteins, wherein the chaperonin proteins can be highlighted. Chaperonins are immunodominant antigens; they are so abundantly expressed that they saturate the immune system with epitopes. Despite the fact that they are highly preserved proteins, they are sufficiently divergent regarding their nucleotide sequence. Only in these last years, studies related to these proteins have addressed the role that they play in the overall virulence processes, mainly in systems of intracellular pathogens. In general, chaperonins are vaccine sources since in addition to being good immunogens they afford protection.

Two degenerated primers were designed from the nucleotide sequences of these preserved peptides for the PCR amplification of the subject protein gene. Once the amplification was carried out, the DNA was isolated and analyzed; the amplified product has 1,137 bp and comprises about the 80 % of the complete gene of the GroEL protein.

Amino acid sequence of CHA.P.s (*Piscirickettsia salmonis* Chaperonin) (SECaa N°1)

DGVSVAKEIELSDKFENMGAQMVKEVASKSNDDAGDGTTTATVLAQAHQE

GVKSVAA

GMNPMDLKRGIDKATIAAVAALKDLSTPCTDNKAIAQVGTISANSDEEIGSH

AKAMEKV

PTDGVITVEEGSSLENELDVVEGMQFDRGYLSPYFVNKQEKMIAEIESPFILLV

DKKISNI

RELLPTLESVAKSGKPLFHAEDVEGEALATLVVNNIRGIVKVCAVKAPGFGD

RRKAMLE

DIAILTGGTVISEEVGLDLEKATLEHLGTAKRIVVTKDNTTVIDGAGEQNAIE

ARVTQIRA

QVEETSSDYDREKLQERVAKLSGGVAVIKVGAATE

Nucleotide sequence of CHA.P.s (*Piscirickettsia salmonis* Chaperonin) (SECan N°1)

GATGGTGTATCTGTTGCCAAAGAAATCGAGCTTAGCGATAAGTTCGAAA

ACATGGG

CGCACAAATGGTCAAAGAAGTCGCATCTAAATCAAATGATGATGCAGG

TGACGGTA

CGACAACGGCGACAGTATTAGCACAAGCAATTATTCAAGAAGGCGTGA

AGTCTGTT

GCTGCCGGCATGAACCCAATGGACCTAAAACGCGGCATCGATAAAGCC

ACTATCGC

TGCAGTTGCTGCATTAAAAGACTTATCTACACCGTGCACAGACAACAAA

GCCATTGC

TCAAGTCGGTACAATTTCAGCAAACTCTGATGAAGAAATTGGCTCTATC

ATTGCTAA

AGCGATGGAAAAAGTACCTACCGACGGCGTAATCACTGTTGAAGAAGG

CTCCAGCC

TTGAAAACGAATTAGATGTTGTTGAAGGGATGCAATTCGATCGCGGTTA

CCTCTCTC

CATATTTTGTCAACAAACAAGAGAAAATGATCGCTGAAATCGAAAGCC

CATTTATCT

TACTCGTCGACAAGAAAATTTCTAACATTCGCGAATTACTACCCACATT

AGAATCAG

TTGCTAAATCAGGCAAGCCATTATTCATCATCGCTGAAGATGTTGAAGG

TGAAGCTC

TGGCAACACTCGTCGTTAATAACATTCGCGGTATTGTTAAAGTGTGCGC

AGTAAAAG

CACCTGGCTTTGGTGATCGTCGTAAAGCGATGCTTGAAGATATTGCCAT

CTTAACTG

GCGGTACTGTAATCTCTGAAGAAGTTGGCCTAGACCTTGAGAAAGCAAC

TCTTGAGC

ACTTAGGTACAGCAAAACGCATCGTCGTCACTAAAGACAATACAACCG

TTATTGATG

GTGCGGGTGAACAAAATGCGATCGAAGCTCGCGTTACTCAAATCCGTGC

ACAAGTT

GAAGAAACATCCTCTGACTACGACCGCGAGAAACTGCAAGAGCGTGTC

GCTAAGCT

ATCTGGTGGTGTTGCTGTCATTAAAGTTGGCGCAGCGACTGAA

This novel protein, which we have denominated CHA.P.s, contains epitopes that allow the protein to act as an excellent immunogen, both *in vivo* and *in vitro*. The CHA.P.s protein was purified from naturally infected fish with *Piscirickettsia salmonis* based on its immunogenic efficiency. In general, chaperonins can be considered as vaccine sources, because aside being good immunogens they afford protection.

In our case, we have already established a protection close to 100% using it as a trial vaccine.

WE CLAIM:

1. A protein CHARACTERIZED by its high immunogenic value against the *Piscirickettsia salmonis* pathogen and obtained naturally by activating the immune system of pathogenic -inoculated animals.

2. A protein according to claim 1, CHARACTERIZED in that it has a molecular mass of 22 kDa.

3. A protein according to claim 1, CHARACTERIZED in that it corresponds to the amino acids sequence SECaa No. 1.

4. A protein according to claim 1, CHARACTERIZED in that it corresponds to the nucleic acids sequence SEC an No. 1.

5. A protein according to claim 4, CHARACTERIZED in that it corresponds to analogues and/or derivatives and/or degenerated nucleic acid sequence of the SEC an No. 1.

6. Recombinant DNA molecule CHARACTERIZED in that it comprises the nucleic acids sequence defined on claims 4 and/or 5 and one o more expression control sequences operatively linked to the nucleic acid sequence.

7. A unicellular host, CHARACTERIZED in that it is transformed with a recombinant DNA molecule according to claim 6.

8. A protein according to claim 1, CHARACTERIZED in that it is alternatively obtained through the culture of a unicellular host, according to claim 7, and the isolation of said protein.

9. A method for preparing a vaccine for preventing the infection of salmons with *Piscirickettsia salmonis*, CHARACTERIZED in that it comprises admixing the protein, according to claim 1, with a pharmaceutically acceptable diluent, excipient or adjuvant.

10. The use of the protein according to claim 1 or a fraction thereof, CHARACTERIZED in that it is intended to manufacture a vaccine for avoiding the infection by the bacteria *Piscirickettsia salmonis* in salmons.

11. A method for preparing a vaccine for preventing the infection of salmons with *Piscirickettsia salmonis*, CHARACTERIZED in that it comprises admixing the protein defined on claim 4, with a pharmaceutically acceptable diluents, excipients or adjuvants.

12. The use of the nucleic acids sequence according to claims 4 or 5 or a fraction thereof, CHARACTERIZED in that it is intended to manufacture a vaccine for preventing the infection of salmons with the *Piscirickettsia salmonis* bacteria.

13. A method for detecting the *Piscirickettsia salmonis* bacteria in a biologic sample, CHARACTERIZED in that it comprises:

a) isolating the biological sample

b) contacting said sample either with (i) the protein defined on claim 1 or (ii) a DNA probe according to the sequence defined on claims 4 or 5

c) detecting the prior bonding of the protein or the DNA probe with the pathogenic , if the latter is present in the biologic sample.

14. The use of the nucleic acid sequence according to claims 4 or 5 or a fraction thereof, CHARACTERIZED in that it is intended to manufacture a molecular marker for the diagnosis of the infection of salmons with the *Piscirickettsia salmonis* bacteria.

PATENT APPLICATION 2086-01

PRELIMINARY EXAMINATION REPLY,

FILING OF A NEW SPECIFICATION

"S.J.D.P.I" (Patent Office Director)

Marino Porzio Bozzollo, Lawyer, patent up to date as authorized by the Municipality of Santiago, with domicile in Santa Lucia No. 330, 7 f., Santiago, on behalf of Universidad Católica de Valparaiso and Alpharma Inc. and based on the background information contained in the present Patent application No. 2086-01, I respectfully submit as follows:

I hereby respond to the report issued by the preliminary examination notified to this party on June 5, 2003, thus accompanying a new Descriptive Brief, which is an integral part of the present Invention Patent Application.

THEREFORE,

I URGE YOU to consider the report issued from the present preliminary examination as having been replied and the referred documents as having been filed, thus progressively attending to the present application.

(one illegible signature)

31/07/03

U.3062/RAV

SPECIFICATION

FIELD OF THE INVENTION

The present invention is, in general, found in the field of molecular biology and more particularly in the area of biotechnology, inasmuch as it mainly describes the form in which to obtain useful tools toward preventing and diagnosing the disease caused by *Piscirickettsia salmonis*; said tools are obtained from a sequence of nucleic acid and a sequence of amino acids.

BACKGROUND OF THE INVENTION

Breeding of live species under captivity to produce meat destined to direct human consumption, as is the case with salmon culture, representing an activity increasingly demanded by consumers and requiring high quality standards, an aspect which is decisive for competing and remaining in domestic and international markets.

The biological subjects which are relevant for the production of salmonid species, have basically been focused on fish reproduction, diseases and feeding. Some aspects obtain almost immediate solutions devised by the producing companies, while others require both basic and advanced scientific research.

Nowadays, Chile is the second world producer of the salmonid species market, with a production and total exports of approximately 240,000 tons of salmon. An aspect of the culture which is essential for the quality control of the final

product and to the profitability of the companies has to do with the diseases which often attack fish population in overcrowded conditions. In view of the importance of this market in our country, the solutions for combating bacterial and viral agents having a strong negative impact on the aquaculture should address the discovery of preventive treatments, such as vaccines.

Early in 1989, the Salmon Rickettsial Syndrome ("SRS") or piscirickettsiosis was described, and since then it has caused significant mortalities in salmon aquaculture. *Piscirickettsia salmonis* is the causative agent of this syndrome, since produces the most important disease affecting the country's salmon-producing system and it translates into a pathology that induces the highest economic losses in Chile. Data supplied by various companies and national entities indicate that the losses resulting from this microorganism reach values close to US$ 80,000,000 (eighty billion UD dollars), which undoubtedly reflect the importance of this bacteria as a pathogenic agent.

The integral control of diseases in salmon, comprises a series of measures that should be jointly applied. The above mentioned involves monitoring, fast and accurate diagnose techniques, alternate use or short-term use of culture locations, a suitable infrastructure offering genetic improvement, optimum hygiene and sanitation of the facilities; nutritional quality of the food, adequate feeding systems, careful handling of the fish ova with a safe origin and a continuous technical capability. Furthermore, by way of important preventive measures against infection, the market offers techniques and products, such as

immunostimulants, hygiene prophylaxis, antibiotics and integral controls of salmon diseases, specially emphasizing on the support to the fish vaccine-based defensive system.

The immunostimulant substances are represented by glucanes, vitamin C and biogenic stimulators. Immunostimulants have been tested recently, therefore, there is no ready market for them yet. Both the hygiene prophylaxis and the diseases integral control systems are substitutes based on prevention-oriented actions; however, they do not guarantee to stop the fish contagion by bacteria and virus.

The only set of concrete curative and non-preventive disease-control products that have always been applied to the salmonidae culture are: Antibiotics; namely, oxolynic acid, erythromycin, flumequine and oxitetracycline.

The sanitary prophylaxis corresponding to those hygiene-sanitary nature measures throughout the whole process chain of salmon production, is based on sanitary measures, using appropriate methods and disinfectants which have a proven action against pathogens, additionally a healthy ova supply is obtained out by way of control.

A National Health Plan elaborated by the Salmon Technological Institute has been implemented in Chile. This Plan is aimed at standardizing and supervising the sanitary handling practices and at obtaining and organizing the information provided by the industry in those areas that are sensitive to the

companies' productivity. Its action lines are the surveillance, prevention, diagnosis and disease control.

All the above-mentioned substitute products are insufficient to ensure the eradication of *Piscirickettsia salmonis* as an obligatory intracellular rickettsial pathogenic because of their failure to reach effective levels for the intracellular location of the infection as well as of the agent. The inconsistent outcome of treatment with antimicrobials has encouraged research into the development of vaccines. The preventive management of diseases through the use of vaccines corresponds to the single viable option, a situation which has been demonstrated by the strategy involving the eradication of antibiotics use and the eradication of other products applied at the domestic level in Norway, thereby provoking the full substitution of the same by vaccines.

Vaccines, in general, have the advantage of being a disease-prevention management approach, whereby they would replace the antibiotics use, and not *vice versa*. Additionally, vaccines have the advantages of a defined efficiency, a significant and proven cost reduction mechanism, long-lasting effects, and a real preventive capacity; thus, additionally, their use overcomes all the disadvantages inherent in the use of antibiotics.

Nowadays, vaccines for *Piscirickettsia salmonis* are commercially available. Thus, *Piscirickettsia salmonis* isolates are now manufactured under the trademark of "Ricketvac"®, which are duly characterized and titrated, often found in the field, propagated in cell cultures and rendered inactive with formaldehyde.

The fact of its dependence from the full bacteria propagation in cell cultures requires having stringent sterility conditions & quality controls; all of them factors which complicate its production management at the industrial level. Additionally, the fact that the pathogenic agent remains at an intracellular stage most of the time and surrounding membrane-type vesicles renders this classical vaccine completely ineffective.

According to the previous state-of-the-art, which evidences difficulties in mass producing vaccines from bacteria isolates as well as an inefficient protection, one of the objectives of the present invention consists in the purification of a highly immunogenic protein, derived from the intracellular pathogenic *Piscirickettsia salmonis*, to ideally promote both a humoral and a cellular response in the fish against the agent.

Another objective of the present invention is to characterize said protein through its amino acid sequence and nucleotide sequence; in turn, said sequences will be useful in obtaining the protein by means of genetic engineering.

A very important objective of the present invention is the development of a vaccine for preventing the *Piscirickettsia salmonis* pathogen infection of the salmonidae species, exemplified but not restricted to the Coho salmon, Atlantic salmon, and the rainbow trout.

Therefore, according to the above, one of the objectives of the invention is to obtain a vaccine, either from the protein itself or from the DNA of said protein.

A further objective is to provide an efficient tool for the detection of *Piscirickettsia salmonis*, which is an important aspect of any integrated control program of the disease caused by this pathogen. In order to do so, a battery of different antibodies against the protein have been elicited in different organisms to promote sensitive detection in the field.

The vaccine, according to the present invention, is distinguished by its high effectiveness in preventing the salmonidae contagion with *Piscirickettsia salmonis*, exhibiting much higher levels as compared with those currently obtained using other products, without exhibiting undesirable side effects, such as adherence.

It may be concluded that there is an ample market for the vaccine against the *Piscirickettsia salmonis*, which can extensively compete with currently existing vaccines, according to what will be demonstrated herein below. Vaccines have no real substitutes. On the contrary, this product will substitute the use of antibiotics, which represent chemical compounds whose soonest strategic removal from salmon culture activities has already been proposed.

DETAILED DESCRIPTION OF THE INVENTION

In an effort to characterize and purify the CHAP protein of the *Piscirickettsia salmonis*, we adopted a combined genomic-proteomic approach based on the following aspects:

i) *Piscirickettsia salmonis* purification: from CHSE-214 tissue culture of infected cells, as close as possible to homogeneity, by means of differential centrifugation of shedded bacteria, followed by DNase I digestion in order to get rid of the contaminant cellular DNA and to purify it by centrifugation of the lodixanol density gradient ;

ii) Production of known immune sera against the purified bacteria, as polyclonal antibodies obtained from rabbits. To be used for detection and characterization purposes;

iii) Identification of the best immunogenic CHAP protein from *in vivo* naturally infected fish, as well as from pathogenic agent-induced infected tissue culture. Differential hydrophobic protein extracts were obtained through a novel method from naturally infected Coho salmon fish organs (liver, kidney and brain) as well as from CHSE-214 infected cells. The resulting proteins were analyzed by uni and bi-dimensional polyacrylamide gels (SDS-PAGE), followed by isoelectric focusing, in order to determine the corresponding isoelectric points of the separated proteins. The Western blot analysis, using the battery of antibodies yielded the most reactive as a surface protein antigen, thereafter named CHAP;

iv) The isoelectric point of the protein resulted being 4.9 and the protein was blotted from the 2-D gel onto an PVDF membrane, in order to be as pure as possible for sequencing purposes. The immunoreactive spot was submitted to micro-sequencing by the standard and well-known Edman degradation

procedure. The salmonid humoral immune system and presumably the cellular one, seems to react strongly to this *P. salmonis* antigen and to afford protection to primary and secondary infection. The purified CHAP protein has a molecular mass of 58.5 kDa and an isolectric point of 4.9.

Once the peptide sequence (Table 1) were obtained, a Basic Local Alignment Search Tool analysis (BLAST) was performed, in order to search for homologous heterologous proteins, which might suggest the potential biological function, as well as the type of CHAP protein represented. The analysis showed that a distinctive homology was observed in the sequenced peptide, although it remained quite specific for *P. salmonis* with the "chaperoning" encoded - groEL genes of proteobacterian species.

Chaperonins are oligomeric molecular chaperones. They belong to the heat shock protein family (HSP's), which are highly preserved and are found in all prokaryotic cells. HSPs are induced by a variety of environmental stresses such as temperature, inflammation, viral and bacterial infections as well as malignant transformation. GroEL genes can be ascribed to the HSP60 chaperonins family, which is best characterized in *E.coli* and which are very similar to the 65 kDa antigen of *Mycobacterium tuberculosis* spp. In both cases they are thought to be immunodominant antigens, which facilitate folding, unfolding and polypeptide translocation, allowing the assembly and disassembly of oligomeric protein complexes. Thus, the protein family has pivotal roles in the normal cell function. Chaperonines, as immunodominant

antigens, are so abundantly expressed that they saturate the epitopes of the immune system. Nonetheless, at the same time as they are highly preserved proteins, they are sufficiently divergent in their nucleotide sequence for providing group-specificity, which means that each chaperonin from each analyzed phylogenetic group is by itself different from its counterparts.

The literature offers a great number of controversies, regarding the roles of molecular chaperones and their role in immunity. This confusion might be due, in part, to the increasing evidence that molecular chaperones are not simply inert immunogens, since they can also participate in the standard immune response activation, as in the case of lymphocytes. Particularly, chaperonins should be classified as 'multiplex antigens' because of their ability to interact with and activate different cells types.

On the basis of the amino acid sequence, obtained for the *P. salmonis* GroEL gene, two degenerated primers B1 and AB4 were designed upon alignment with others groEL gene sequences in order to promote its amplification from the purified *P. salmonis* genome. These primers cover 70% of the total gene length, a PCR was performed using primer sets as indicated in Table 2, under optimal standardized cloning conditions. The resulting DNA amplifications were cloned into a TOPO-TA expression vector. The expression and purification of the cloned gene, was attained using the "PurePro Caulobacter Expression System" a new protein production system, from Invitrogen, based

on the *Caulobacter crescentus* bacterium, which delivers the pure protein into the growing media, thus simplifying its recovery.

Upon confirmation of the specificity sequence of the expressed truncated protein (70%), a new CHAP BLAST alignment was carried out in order to design the degenerated primers set to cover the full length of said novel *P.salmonis* protein (Table 2, FIG. 1).

The full length of the CHAP protein amplified under standard conditions with the new primers CHAP11 and CHAP8, was also cloned in the pCXTOPO-TA expression vector. The plasmid carrying the recombinant gene, encoding the fusion protein, was introduced into *Caulobacter crescentus* via electroporation. The electro-transformants were selected and grown on an expression medium for another 72- 96 hours. The aggregated fusion protein, was recovered from the culture medium, through sieving on a nylon mesh, it was rinsed and re-suspended to formulate the vaccine.

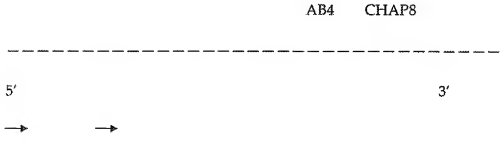

TABLE 1: Amino acid residues deduced from the CHAP protein

Peptide sequence	Peptide no.
SFGAPTITK	43-51
FENMGAOMVK	66-75
VAAGMNPMDLKR	107-118
ELLPTLESVAK	232-242
AAVEEGVVPGGGVALVR	405-421

TABLE 2: Oligonucleotide primers used for PCR

Primer	Sequence (5' to 3')	Position[a]
Forward	[b]	
CHAP 11	GGAGATATAAGAA**ATG**TCAGCAAAAGAAGTG	1-18
B1	GTCHTTCGGYGCDCCRACCATYAC	126-149
Reverse		
CHAP 8	CATCATRCCGCCCATKCCRCCCAT	1612-1635
AB4	CCRCCYGGWACVACRCCTTCTTC	1244-1222

[a] relative Position to *P. salmonis* GroEL genomic sequence, disclosed in this application

[b] Starting methionine

Figure 1, corresponds to a schematic diagram of the DNA fragment, which codifies the GroEL gene of *P. salmonis* , amplified by PCR from the genomic DNA of *P. salmonis*; The numbers are the nucleotide position regarding the sequence that is disclosed in the present application.

Aminoacidic sequence of CHAP (*Piscirickettsia salmonis* Chaperonin)

(SEC aa Nº1)

.....

Nucleotide sequence of CHAP (*Piscirickettsia salmonis* Chaperonin)

(SEC an Nº1)

.....

WE CLAIM:

1. A protein CHARACTERIZED in that it is provides immunogenicity against *Piscirickettsia salmonis* pathogen, naturally obtained through the activation of the immune system of fish inoculated with the pathogen.

2. A protein according to claim 1, CHARACTERIZED in that it has a molecular mass 58.5 kDa and an isoelelctric point of 4.9.

3. A protein according to claim 1, CHARACTERIZED in that it corresponds to the amino acid sequence SECaa No. 1.

4. A protein according to claim 1, CHARACTERIZED in that it corresponds to the nucleic acids sequence SEC na No. 1.

5. A protein according to claim 1, CHARACTERIZED in that it corresponds to analogues and/or derivatives and/or degenerated sequences of nucleic acids sequences SEC na No. 1.

6. A protein homologous to the GroEL protein, according to claim 1, which is derived from other bacteria species exemplified by but not excluding *E. coli, M. tuberculosis*, etc., and the use of the same in a vaccine for fish and other marine organisms.

7. A recombinant DNA molecule, CHARACTERIZED in that it comprises the nucleic acid sequence defined on claims 4 and/or 5, and one or more expression control sequences operatively linked to the nucleic acid sequence.

8. A unicellular host, CHARACTERIZED in that it is transformed with a DNA recombinant molecule according to claim 6.

9. A protein or a peptide according to claim 1, CHARACTERIZED in that it is alternatively obtained through the culture of a unicellular host, according to claim 7, and the isolation of said protein.

10. A method for preparing a vaccine for preventing the infection of fish with *Piscirickettsia salmonis*, wherein said fish are not restricted to the salmonid species, such as to Coho salmon, Atlantic salmon, Rainbow trout or non-salmonid species, for example but not restricted to White seabass, Black seabass, Tilapia, CHARACTERIZED in that it comprises a mixture of the protein according to claim 1, with a pharmaceutically acceptable diluent, excipient or adjuvant.

11. The use of the protein according to claim 1 or a fraction thereof, CHARACTERIZED in that it is intended to manufacture a vaccine, for

preventing the infection of fish with the *Piscirickettsia salmonis* bacteria, wherein said fish are not restricted to salmonid species, such as to Coho salmon, Atlantic salmon, Rainbow trout or non-salmonid species, exemplified but not restricted to, White seabass, Black seabass and Tilapia.

12. A method for preparing a vaccine for preventing the infection of fish with *Piscirickettsia salmonis*, i.e in salmonid exemplified species, but not restricted to Coho salmon, Atlantic salmon and Rainbow trout or non-salmonide, exemplified but not restricted to White seabass, Black seabass, Tilapia, CHARACTERIZED in that it comprises the steps of mixing the protein according to claim 4, with a pharmaceutically acceptable diluent, excipient or adjuvant.

13. A method for preparing a vaccine, for preventing the infection of fish with *Piscirickettsia salmonis*, i.e in salmonid species, exemplified but not restricted to Coho salmon, Atlantic salmon, Rainbow trout or non-salmonide, exemplified but not restricted to White seabass, Black seabass, and Tilapia in combination with antigens which protect against other bacterial and viral diseases in the fish, exemplified but not restricted to *Aeromonas salmonicida*, *Vibrio anguillarum*, *Renibacterium salmoninarum*, *Yersinia ruckeri*, Infectious pancreas necrosis virus, Infectious salmon anemia virus, CHARACTERIZED in that it comprises the steps for mixing the protein according to claim 4 or fragments thereof, with other antigens in a pharmaceutically acceptable diluent, excipient or adjuvant.

14. The use of a sequence of nucleic acids as described in claims 4 or 5 or a fraction thereof, CHARACTERIZED in that is intended to manufacture a vaccine for preventing the infection of fish with the *Piscirickettsia salmonis* bacteria, i.e salmonides, exemplified but not restricted to Coho salmon, Atlantic salmon, Rainbow trout or non-salmonides, exemplified but not restricted to White seabass, Black seabass, and Tilapia.

15. A method detecting the *Piscirisckettsia salmonis* bacteria in a biological sample, CHARACTERIZED in that it comprises the following steps:

(a) isolating the biological sample;

(b) contacting said sample either with (i) the protein according to 1, or (ii) a DNA probe according to the sequence of claims 4 or 5;

(c) detecting the previous binding of the protein or the DNA probe with the pathogenic, if the latter is present in the biological sample.

16. The use of the sequence of nucleic acids as described in claims 4 or 5 or a fraction thereof, CHARACTERIZED in that it is intended to manufacture a molecular marker for the diagnosis of the infection of fish with the *Piscirickettsia salmonis* bacteria such as salmonides, exemplified but not restricted to Coho salmon, Atlantic salmon and Rainbow trout or non-salmonide, exemplified but not restricted to White seabass, Black seabass, and Tilapia.